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PRINCIPAL INVESTIGATOR: Yihong Wan  
Steven K. Nordeen, Ph.D.

CONTRACTING ORGANIZATION: University of Colorado Health  
Sciences Center  
Denver, Colorado 80262

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Colorado Health Sciences Center Denver, Colorado 80262 E-Mail: yihong.wan@uchsc.edu Steve.nordeen@uchsc.edu		8. PERFORMING ORGANIZATION REPORT NUMBER		
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## Introduction

Progestins and glucocorticoids are two classes of steroid hormones with very distinct biological functions. The major physiological role of progestins in the mammal are to establish and maintain pregnancy in the uterus and ovary; to promote lobular-alveolar development of the mammary gland and to suppress milk protein synthesis during pregnancy. Some classical biological actions of glucocorticoids are regulation of metabolism, suppression of bone formation, and inhibition of inflammation and the immune system. In the pathology of breast cancer, it has been shown that progestin agonist can increase the incidence of spontaneous mammary tumors. Some studies indicated that physiological doses of progestin agonists can stimulate the growth of established tumors, while high pharmacological dose progestins can inhibit the growth of established tumors (1). However, there is no evidence of a role of glucocorticoids in breast cancer even though both progesterone receptor and glucocorticoid receptor are expressed in the mammary gland. Interestingly, progesterone receptor (PR) and glucocorticoid receptor (GR) share many similar structural and functional characteristics: similar amino acid sequences and functional domains; similar, if not identical, DNA sequence recognition specificity; associate with a similar complex of molecular chaperones in the absence of hormone and with a similar set of coactivators or corepressors in the presence of hormone. How can two receptors with such remarkable similarity mediate such dramatically different biological functions? We hypothesize that one way that progestins and glucocorticoids can exert different biological effects is through their different abilities to regulate the expression of certain target genes. In order to understand the role of progestins in breast cancer and how progesterone receptor mediates this hormone specific regulation, we identified genes that are differentially regulated by progestins and glucocorticoids in the human breast cancer cell line T47D/A1-2 using two systematic strategies: a retroviral promoter-trapping strategy and an Affymetrix oligonucleotide microarray analysis. The long-term goal of this investigation is to understand the mechanisms of the differential hormone regulation. The genes identified in this investigation play an important role in hormone functional specificity. Knowledge gained from this investigation will be fundamental to the understanding of both hormone actions and their roles in breast cancer.

## Body

**AIM ONE:** Develop a retroviral promoter-trapping strategy to identify promoters/enhancers differentially induced by progestins and glucocorticoids in the human breast cancer cell line T47D/A1-2.

As reported in the 2000 annual summary, a *cre/lox* retroviral promoter-trapping system was established in a mouse fibroblast cell line, 4F, that express both GR and PR. Using sequential-selection screening strategies, clonal cell lines representing genes potentially regulated or differentially regulated by the two hormones were isolated.

To determine if the gene represented by each clonal cell line is indeed regulated by hormone, we developed a reporter assay to quantitate the Cre protein level based on Cre recombinase activity (Fig. 1A). Luciferase expression from plasmid ppgklxtkneo/luc was dependent on Cre expression. A titration of Cre expression vector demonstrated that the higher the input of Cre, the higher the output of luciferase expression (Fig. 1B). Cell clones represent candidate, hormone-regulated promoters were transfected with ppgklxtkneo/luc and treated with glucocorticoids or progestins 24 hrs later. The induction of Cre activity was assessed by the deletion of sequences flanked by *loxP* sites as measured by the appearance of luciferase activity. In 32 cell lines, luciferase expression was induced by either hormone by more than 2 fold. In 19 cell lines, luciferase expression was differentially regulated by glucocorticoids and progestins by more than 1.5 fold. Fig. 1C illustrates the results for selected clonal cell lines. Six clonal cell lines were selected for further analysis (clones 22, 32, 42, 43, 55 and 66).

In order to determine whether the endogenous cellular gene trapped in each of the selected clonal cell lines is hormone regulated, sequences upstream of the proviral integration site were retrieved by inverse PCR and sequenced (2). All sequences showed a typical cellular DNA-provirus junction (2). Extensive database searches revealed no homology to known genes or expressed sequence tags, suggesting that these are novel hormone-regulated genes. Semi-quantitative RT-PCR was performed to determine if the endogenous gene is hormone-regulated (Fig. 2). Clone 32 gene was preferentially induced by glucocorticoids (5 fold) than by progestins (2.5 fold). Clone 43 gene was also preferentially induced by glucocorticoids. Clone 42 gene was induced by both hormones to a similar level. These results were in agreement with the Cre protein regulation patterns shown in Figure 1C. The genes in clones 22 and 55 were induced by neither hormone. This contrasts with the Cre protein regulation pattern in these clonal cell lines, possibly due to hormone regulation of Cre expression at post-transcriptional levels. The primers designed for the upstream sequences of clone 66 did not give RT-PCR product even though they can amplify the genomic DNA (data not shown). The integration site in this clone may not be in exonic sequence or the 5' primer could be upstream of the transcription start site.

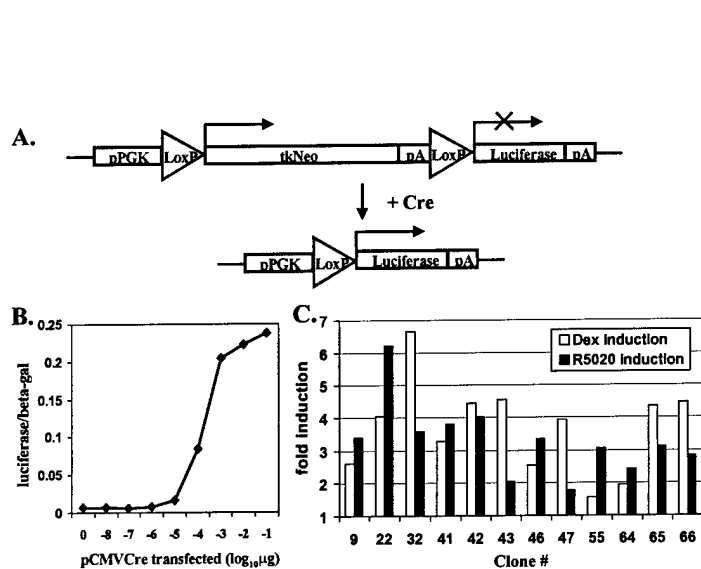
Expression of clone 32 gene was further analyzed by semi-quantitative RT-PCR. A time course demonstrated that glucocorticoid induction of clone 32 gene peaked as early as 30 min, indicating this is a direct response of hormone regulation. The hormone induction lasted for at least 22 hrs (Fig. 3A). The hormone regulation of clone 32 gene was assessed in two other cell types. L929 cells are also mouse fibroblasts like the 4F cells. L929 cells express only GR but not PR. Clone 32 gene was also induced by glucocorticoids in L929 cells (Fig. 3B). T47D/A1-2 cells are human breast cancer derived cells and expresses both GR and PR. Clone 32 gene was induced by neither glucocorticoids nor progestins in these cells (Fig. 3C). These results indicate that the hormone regulation of clone 32 gene is cell-type specific.

In summary, this retroviral promoter trapping system allowed the identification of two novel genes that are differentially regulated by glucocorticoids and progestins. Another novel gene was identified to be induced by both hormones. Future studies on the mechanism of the differential gene regulation will enhance our understanding of hormone-specific actions.

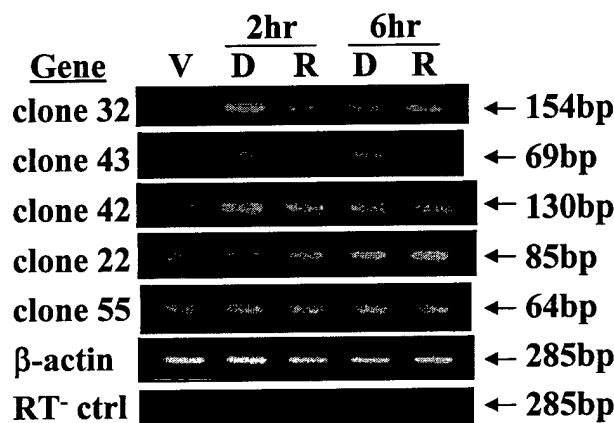
**AIM TWO** Implement Affymetrix oligonucleotide array analysis to identify genes differentially regulated by progestins and glucocorticoids in the human breast cancer cell line T47D/A1-2.

T47D/A1-2 cells were treated with vehicle, dexamethasone ( $10^{-7}$ M) or R5020 ( $10^{-8}$ M) for 2hrs or 6hrs. Total RNA was harvested. cRNA probes were synthesized and hybridized to the HugenFL oligonucleotide chip array. Of 5600 genes analyzed, 65 were upregulated by glucocorticoids by more than 3 fold at either 2hr or 6hr treatment, and another 26 were downregulated. The number of genes that are upregulated and downregulated by progestins are 45 and 30, respectively. In particular, 42 genes were identified to be differentially regulated by more than 2 fold (Fig. 4). Four of the differentially regulated genes representing different regulation pattern were chosen to be confirmed by RT-PCR (data not shown) and quantitated by northern blot analysis (Fig. 5). The **G0S8** gene was originally identified as a G0/G1 switch gene in human lymphocytes (3). It is also known as RGS2 (Regulator of G protein Signaling 2) and encodes a Gq $\alpha$  inhibitor(4). It is a helix-loop-helix basic phosphoprotein (3). G0S8 knockout mice show reduced T cell proliferation and antiviral immunity, increased anxiety responses and decreased male aggression (5). Quantitation of the northern blot confirmed the array analysis and demonstrated that G0S8 was induced by glucocorticoids by 20 fold, but not by progestins (Fig. 5A). The **INHBB** gene encodes the beta-B subunit of inhibins and activins, which are members of the TGF $\beta$  superfamily. Inhibins and activins modulate the growth and differentiation of several cell types. INHBB knockout mice have lactation failure due to retarded ductal elongation, alveolar morphogenesis and milk secretion (6). In contrast to G0S8, both of the two transcripts for INHBB were induced dramatically by progestins, while glucocorticoids treatment led to no induction or even downregulation (Fig. 5B). The **PLZF** (Promyelocytic Leukemia Zinc Finger) gene was originally identified as a partner gene fused to RAR $\alpha$  in a variant chromosomal translocation in APL (7). It encodes a nuclear transcription factor of the BTB/POZ family (8). Both of the two transcripts for PLZF were induced by glucocorticoids at three times higher fold than by progestins (Fig. 5C). The **IEX-1** gene was identified as a UV inducible immediate-early gene in human keratinocytes (9, 10). It is also known as Dif-2 and is downregulated during monocyte differentiation (11). It can be induced by multiple signals, e.g. LPS, C<sub>2</sub>-ceramide, lysoPC and PMA (11). Glucocorticoid treatment led to downregulation of IEX-1 gene expression by 80%, while progestin treatment only had a transient and weak effect (Fig. 5D). Treatment with a protein synthesis inhibitor or receptor antagonist indicated that the hormone regulation is protein synthesis independent but receptor dependent (data not shown).

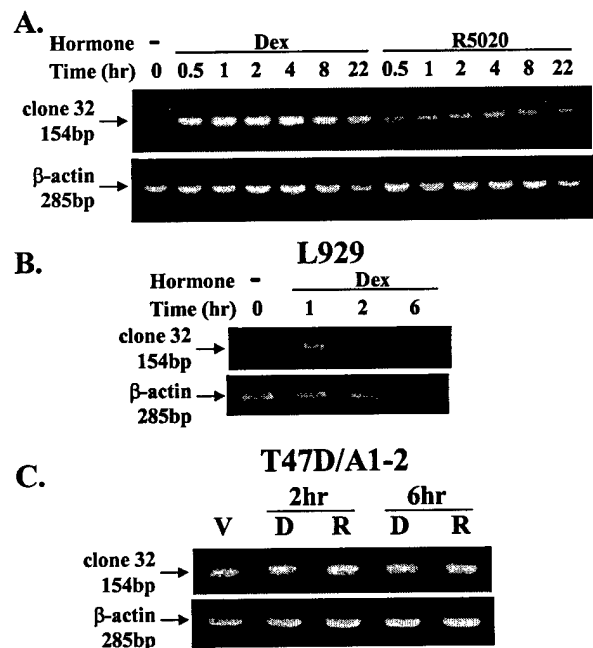
In summary, the Affymetrix microarray analysis allowed the identification of a group of hormone differentially regulated genes in the human breast cancer cell line T47D/A1-2. Confirmation of the expression pattern of all four genes selected with RT-PCR and Northern blot analysis demonstrated the reliability of the array results. Analysis of the mechanisms underlying the expression pattern of these genes will help us to understand differential regulation by the two hormones and the specific role of progestins in breast cancer.



**Fig. 1. Quantitation of Cre protein expression in selected cell lines.** **A.** Schematic of ppgklxtkneo/luc reporter construct before and after Cre-mediated recombination at *loxP* sites. pPGK, mouse phosphoglycerate kinase promoter; tkneo, herpes simplex virus 2 thymidine kinase (*tk*)-neomycin phosphotransferase (*neo*) fusion gene; pA, a pair of polyadenylation signals; *loxP*, Cre recombination target sequences. **B.** A dose curve demonstrating that relative levels of Cre activity can be analyzed by luciferase activity. Increasing amounts of the expression plasmid pCMVCre was transiently transfected into the 4Fneo/hygro21 cell line. Constant amount of the reporter tkneo/luc (1 µg/ml) and an internal control plasmid pCMVβ-gal (0.1 µg/ml) were cotransfected. Cells were harvested 72 hrs later. The result was shown as luciferase activity normalized by β-galactosidase activity. **C.** Plasmid ppgklxtkneo/luc (1 µg/ml) was transiently transfected into the indicated clonal cell lines with an internal control plasmid pCMVβ-gal (0.1 µg/ml). After 20-24 hrs, cells were treated with vehicle, dexamethasone (Dex, 100 nM) or R5020 (10 nM). Cells were harvested 48 hrs after treatment. The results were shown as the fold of induction of the luciferase activity normalized to β-galactosidase activity.



**Fig. 2. Quantitation of the mRNA level of genes represented by clone 32, 43, 42, 22 and 55 in 4F cells by semi-quantitative RT-PCR.** Parental cells (4F) were treated with vehicle (V), dexamethasone (D, 100 nM) or R5020 (R, 10 nM) for 2hr or 6hr. Total RNA was isolated and used for semi-quantitative RT-PCR.



**Fig. 3. Glucocorticoid induction of clone 32 gene is an early response and is cell-type specific.** **A.** The kinetics of the hormone regulation of clone 32 gene. 4F cells were treated with dexamethasone (Dex, 100 nM) or R5020 (10 nM) for the indicated time. Total RNA was isolated and used for semi-quantitative RT-PCR. **B.** **C.** Hormone regulation of clone 32 genes in different cell lines. L929 cells (**B**) and T47D/A1-2 cells (**C**) were treated with vehicle (V), dexamethasone (D or Dex, 100 nM) or R5020 (R, 10 nM) for indicated time. Total RNA was isolated and used for semi-quantitative RT-PCR.

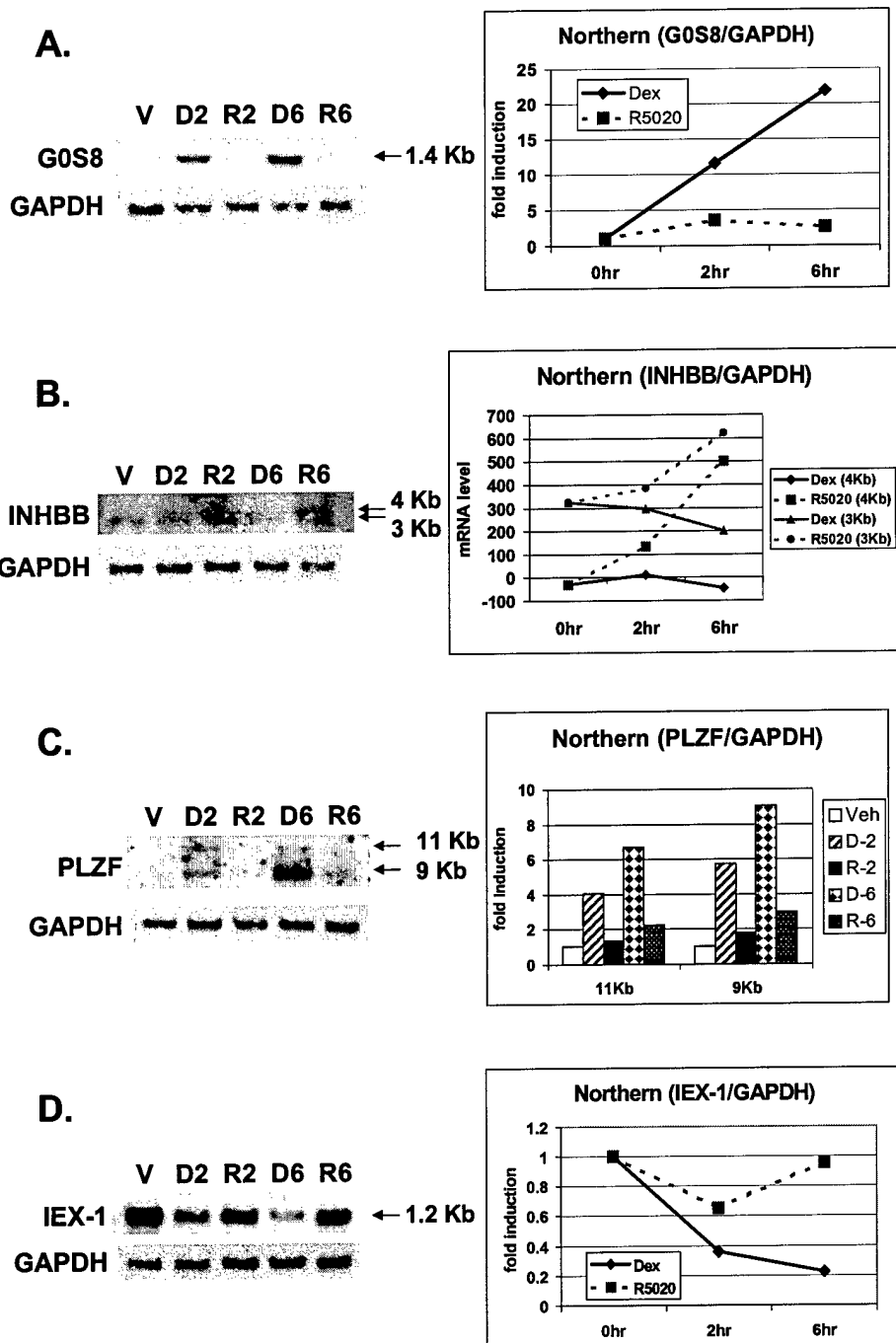
**Fig. 4. Summary of genes identified to be differentially regulated by glucocorticoids and progestins by more than 2 fold**

Probe Set	Description	Fold Diff.*		Fold	Change	
D>R		D : R	D2 :V	D6 :V	R2 :V	R6 :V
L13391_at	HLH basic phosphoprotein (G0S8)	15.4	8.5	14.3	2.9	-1
Z19002_at	PLZF, kruppel-like zinc finger protein	9.6	7.3	14.4	2.9	4.5
S73591_at	brain HHCPA homolog	5.1	1.2	1.1	-2.3	-4.5
X17059_s_at	NAT1, arylamine N-acetyltransferase	4.5	1.4	2.1	-1.4	-2.1
D89377_s_at	MSX-2 homeoprotein	4.4	1.7	2.8	-1	-1.5
X77777_s_at	intestinal VIP receptor related pr.	4.3	3.8	10.7	-1.1	1.7
U48251_at	protein kinase C-binding protein RACK7	3.9	1.5	1.6	1.8	-2.5
HG3494-HT3688_at	nuclear factor Nf-II6	3.8	1	1.4	-1	-2.6
HG4310-HT4580_at	cellular retinol binding protein Li	3.8	3.6	10.7	1.5	7.3
X52730_rna1_at	phenylethanolamine N-methylase (PNMT)	2.7	1.2	2.5	-1	-1.1
U37518_at	TNF-related apoptosis inducing ligand TRAIL	2.7	-1.3	-2.9	1.3	-6.2
D87953_at	RTP (tunicamycin-responsive protein)	2.3	1.3	2.6	1	1.1
D<R						
M74089_at	TB1 gene (related to colorectal cancers)	-10.5	-8	1.2	1.3	1.2
M29874_s_at	cytochrome P450-IIIB(hIIBI)	-7.8	-8.7	-2	-1.3	-1.9
M21121_at	T-cell specific protein (RANTES)	-6.4	-2.6	-1.4	2.5	-2.7
U48807_at	MAP kinase phosphatase (MKP-2)	-5.7	1.3	-3.6	1.1	1.6
M80359_at	p78 protein, Interferon, virus induced gene	-5	-3.4	1.1	1.9	1.4
U23070_at	putative transmembrane protein (nma)	-5	1	-4.2	1.9	1.2
M26311_s_at	cystic fibrosis antigen gene	-4.3	-1.8	-1.4	2.4	-3
M31682_at	testicular inhibin beta-B subunit	-4.3	2.4	1.9	4.9	8.3
S81914_at	IEX-1= radiation-inducible immediate-early gene	-4.1	-4.3	-7.6	-2.3	-1.9
M59807_at	NK4 (IL-2 induced gene in NK cells)	-4	-2.1	-1.4	1.7	-2.2
M88163_at	global transcription activator homolog	-3.7	-2.8	1.1	1.4	1
M81118_at	alcohol dehydrogenase chi polypeptide (ADH5)	-3.7	-3.1	1.2	1.2	-1
U22970_rna1_s_at	interferon-inducible peptide (6-16)	-3.7	-1.8	1.5	2	1.1
L13210_at	Mac-2 binding protein	-3.5	-2.1	-1.5	1.7	-9.3
U03688_at	dioxin-inducible cytochrome P450 (CYP1B1)	-3.4	-1.4	-2.5	-1.3	1.4
HG110-HT110_s_at	Heterogenous Nuclear Ribonucleoprotein A/B	-3.4	-4.8	1.1	-1.4	1.3
M92357_at	B94 protein (TNFalpha inducible)	-3.2	-2.3	-5.5	1.7	-5.5
M64174_at	protein-tyrosine kinase (JAK1)	-3.1	-2.5	-1.2	1.3	-1.2
M57730_at	B61 gene, cytokine inducible secretory protein	-2.8	-2.8	1.2	1	1.1
M13755_at	interferon-induced 17-Kda/15-Kda pr.	-2.7	-1.6	1	2	-1.6
M24594_at	interferon-induced 56K protein	-2.7	-1.6	-2.2	1.7	-2.5
M62762_at	vacuolar H+ ATPase proton channel subunit	-2.5	-2.4	1	1.1	1.1
M97936_at	transcription factor ISGF-3	-2.5	-1.5	-1.9	1.6	-2.6
L15702_at	complement factor B	-2.5	-1.2	-1.2	2.1	-1.3
M83751_at	arginine-rich protein (ARP)	-2.5	-2.3	-1.1	1.1	1.1
M87434_at	71 kDa 2'5' oligoadenylate synthetase	-2.5	-2	-1.8	1.3	-2.7
L38490_s_at	ADP-ribosylation factor	-2.3	-1.5	2	1.5	3.7
M33336_at	cAMP-dependent protein kinase type I-alpha subunit	-2.3	-2	1	1.2	1
HG3510-HT3704_at	V-Erba Related Ear-3 protein	-2.2	-1.2	-2.6	-1.1	-1.1
M83088_at	phosphoglucosyltransferase 1 (PGM1) gene	-2.2	-2.2	-1.3	1	1
Controls						
U26726_at	11-beta-hydroxysteroid dehydrogenase type 2	1.3	7.4	27.1	6.9	21
X00351_f_at	beta-actin	-1.4	1	-1.3	-1.1	1.1
U37689_at	RNA pol II subunit	1.2	1.1	-1.1	-1.1	1

Abbreviation: D, dexamethasone; R, R5020; V, vehicle; D2; dexamethasone 2hr; D6, dexamethasone 6hr; R2, R5020 2hr; R6, R5020 6hr

\*. Fold difference shown here is the higher fold difference between 2hr and 6hr treatment





**Fig. 5 Northern blot analysis.** T47D/A1-2 cells were treated with Veh (V), dexamethasone (D or Dex, 100 nM) or R5020 (R, 10 nM) for 2 hrs (D2 or R2) or 6 hrs (D6 or R6). Total RNA was isolated and used for Northern blot analysis. Northern blot membranes were hybridized to cDNA probes for (A) G0S8, (B) INHBB, (C) PLZF or (D) IEX-1. For RNA loading control, each membrane was stripped and hybridized to a cDNA probe for GAPDH. The Results were quantitated by phosphoimager and are shown in the right panel of each corresponding northern blot image.

## **Key Research Accomplishments**

### **Aim One:**

- Using the retroviral-promoter trapping system we established, we isolated 19 cell lines representing genes that are potentially differentially regulated by glucocorticoids and progestins by more than 1.5 fold; we also isolated 32 cell lines representing genes that are potentially induced by either hormone by more than 2 fold.
- For 6 of the clonal cell lines selected, the genomic DNA upstream of the retroviral integration sites were retrieved. Sequence analysis and database search indicated that these are novel genes.
- By assessing the mRNA level of the endogenous genes identified, two novel genes were confirmed to be differentially regulated by glucocorticoids and progestins. Another novel gene was confirmed to be induced by both hormones.

### **Aim Two:**

- In the Affymetrix microarray analysis, of 5600 genes analyzed, 65 were upregulated by glucocorticoids by more than 3 fold at either 2hr or 6hr treatment, and another 26 were downregulated. The number of genes that are upregulated and downregulated by progestins are 45 and 30, respectively. In particular, 42 genes were identified to be differentially regulated by more than 2 fold.
- The expression patterns of four of the differentially regulated genes selected were all confirmed by RT-PCR and northern blot analysis, demonstrating the reliability of the array results.
- Treatment with a protein synthesis inhibitor or receptor antagonist indicated that the hormone regulation of the genes identified is protein synthesis independent but receptor dependent.

## **Reportable Outcomes**

### **Aim One:**

- Wan Y and Nordeen SN (Manuscript submitted to Journal of Molecular Endocrinology) Identification of genes differentially regulated by glucocorticoids and progestins using a Cre/loxP mediated retroviral promoter-trapping strategy.

### **Aim Two:**

- Wan Y and Nordeen SK (2001) Microarray analysis of differential gene regulation by glucocorticoids and progestins in human breast cancer cells. Oral presentation, 83<sup>rd</sup> Annual Meeting of The Endocrine Society, Denver, CO
- Wan Y and Nordeen SN (Manuscript in preparation) Microarray analysis of differential gene regulation by glucocorticoids and progestins in human breast cancer cells.

## Conclusions

The retroviral promoter trapping system allowed the identification of two novel genes that are differentially regulated by glucocorticoids and progestins. Another novel gene was identified to be induced by both hormones. The Affymetrix microarray analysis allowed the identification of 42 hormone differentially regulated genes in the human breast cancer cell line T47D/A1-2. Confirmation of the expression pattern of all four genes selected with RT-PCR and Northern blot analysis demonstrated the reliability of the array results. Analysis of the mechanisms underlying the expression pattern of these genes identified will help us to understand differential regulation by the two hormones and the specific role of progestins in breast cancer.

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## Appendices N/A